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# Structural characterization of NDH-1 complexes of *Thermosynechococcus elongatus* by single particle electron microscopy

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## Abstract

The structure of the multifunctional NAD(P)H dehydrogenase type 1 (NDH-1) complexes from cyanobacteria was investigated by growing the wild type and specific *ndh* His-tag mutants of *Thermosynechococcus elongatus* BP-1 under different CO<sub>2</sub> conditions, followed by an electron microscopy (EM) analysis of their purified membrane protein complexes. Single particle averaging showed that the complete NDH-1 complex (NDH-1L) is L-shaped, with a relatively short hydrophilic arm. Two smaller complexes were observed, differing only at the tip of the membrane-embedded arm. The smallest one is considered to be similar to NDH-1M, lacking the NdhD1 and NdhF1 subunits. The other fragment, named NDH-1I, is intermediate between NDH-1L and NDH-1M and only lacks a mass compatible with the size of the NdhF1 subunit. Both smaller complexes were observed under low- and high-CO<sub>2</sub> growth conditions, but were much more abundant under the latter conditions. EM characterization of cyanobacterial NDH-1 further showed small numbers of NDH-1 complexes with additional masses. One type of particle has a much longer peripheral arm, similar to the one of NADH: ubiquinone oxidoreductase (complex I) in *E. coli* and other organisms. This indicates that *Thermosynechococcus elongatus* must have protein(s) which are structurally homologous to the *E. coli* NuoE, -F, and -G subunits. Another low-abundance type of particle (NDH-1U) has a second labile hydrophilic arm at the tip of the membrane-embedded arm. This U-shaped particle has not been observed before by EM in a NDH-1 preparation.

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**Keywords:** NDH-1; Complex I; *Thermosynechococcus elongatus*; Electron microscopy

## 1. Introduction

Cyanobacteria form a large and widespread group of photoautotrophic micro-organisms, which originated, evolved, and diversified early in Earth's history. The earliest forms attributed to this group were found in sedimentary rocks formed 3.5 billion years ago, and it is commonly accepted that cyanobacteria played a crucial role in the Precambrian phase by contributing oxygen to the atmosphere. Polyphasic taxonomies of cyanobacteria, integrating both phenotypic and genotypic characters is currently being assembled. A milestone in the study of cyanobacteria was the publication of the entire genome of the non-

nitrogen-fixing unicellular cyanobacterium *Synechocystis* sp. PCC 6803 [1]. A number of other cyanobacterial genome projects have been completed. Cyanobacteria may possess several enzymes that are directly involved in hydrogen metabolism: nitrogenase(s) catalyzing the production of hydrogen (H<sub>2</sub>) along with the reduction of nitrogen to ammonia, an uptake hydrogenase catalyzing the consumption of hydrogen produced by the nitrogenase, and a bidirectional hydrogenase which has the capability to both take up and produce hydrogen.

Furthermore an incomplete version of respiratory complex I (11 subunits out of 14 strictly conserved in other prokaryotes like *E. coli*) was identified in cyanobacteria, and it was suggested that the bidirectional hydrogenase fulfils the functions of the missing subunits [2]. However, other data do not support this theory such as the fact that the bidirectional enzyme is not a universal

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cyanobacterial enzyme [3]. Since the bidirectional hydrogenase is absent in a significant set of strains, it seems unlikely that it plays a common, central role in cyanobacterial respiratory complex I, unless different cyanobacteria have adopted different strategies. Some of the conserved sequence motifs of the bidirectional hydrogenase are similar in the two corresponding complex I subunits, but apart from this there are only low sequence similarities. Some authors proposed that the cyanobacterial and chloroplastidial complex, which both have 11 subunits in common with respiratory complex I, might work as a NADPH:plastoquinone oxidoreductase, possibly involved in cyclic photosynthetic electron transport, and that sequence similarities observed between the NADH dehydrogenase part of complex I and the bidirectional hydrogenase are due to a common ancestor [4]. It should be pointed out that, at present, the existence of a 15-subunit respiratory complex I in cyanobacteria cannot be ruled out. For instance, the *Nostoc* strain PCC 73102, which lacks the bidirectional hydrogenase, respire at rates comparable to those of other cyanobacteria, and bidirectional hydrogenase-minus mutants of *Synechocystis* strain PCC 6803 exhibited growth and respiration rates comparable to those of the wild type [5]. Obviously more data are necessary to clarify the function of the bidirectional hydrogenase in cyanobacteria.

Reverse genetics has been essential in revealing the roles of specific Ndh subunits [6]. NDH-1 complexes containing NdhD1 and NdhD2 proteins, together with NdhF1, have been postulated to function in PSI-associated cyclic electron flow as well as in cellular respiration [7–9]. NDH-1 complexes with other NdhD and NdhF gene products have been suggested to have additional functions in carbon concentrating mechanism in cyanobacteria [3,6,9–12]. These mechanisms are important in aquatic organisms to overcome the low affinity of their ribulose-1,5-bisphosphate carboxylase/oxygenase to CO<sub>2</sub> [13,14]. Four distinct inorganic carbon acquisition systems have been identified in cyanobacteria by reverse genetics approaches [15]. Two of them are specialized in CO<sub>2</sub> uptake [3,9,12]. One is a constitutively expressed low-affinity CO<sub>2</sub> uptake system, and the other is a high-affinity CO<sub>2</sub> uptake system induced at limiting CO<sub>2</sub> conditions. Reverse genetics with cyanobacteria has demonstrated that the inducible CO<sub>2</sub> uptake system involves the NdhD3 and NdhF3 proteins, whereas the constitutively expressed CO<sub>2</sub> uptake system involves NdhD4 and NdhF4 proteins [3,9,12]. Moreover, the two homologous proteins CupA and CupB are essential for inducible and constitutive CO<sub>2</sub> uptake, respectively [3,12]. Their function in CO<sub>2</sub> uptake systems has been suggested to occur via specialized NDH-1 complexes [9]. The other two inorganic carbon acquisition systems in cyanobacteria are involved in bicarbonate transport.

Although considerable progress has been made during the past few years in elucidating the functions of the NDH-1 complexes in cyanobacteria and chloroplasts, the structural basis and cooperation of various complexes still requires elaborate research. The low-resolution structure of mitochondrial Complex I from several organisms was determined by (cryo-) electron microscopy of negatively stained and unstained specimens [16–20], but currently no 2D maps or 3D structure are available for NDH-1 from cyanobacteria. We have performed an

electron microscopy study of the various NDH-1 complexes, purified under low- and high-CO<sub>2</sub> growth conditions. We have characterized at least four different types of NDH-1 complexes in our preparation and based on a previous proteomic characterization of the various types of purified particles [21] we draw conclusions about their subunit composition.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The construction of *Thermosynechococcus elongatus* NdhL-His strain was described in [21]. WT and NdhL-His strains were grown in BG-11 medium at 45 °C under 50 μmol photons m<sup>-2</sup> s<sup>-1</sup> in 1 L batch cultures under gentle agitation at high CO<sub>2</sub> (4.5% CO<sub>2</sub> in air) or low CO<sub>2</sub> (air level). The medium for growing the NdhL-His strain was supplemented with 3.4 μg/ml chloramphenicol.

### 2.2. Purification of NDH-1 complexes

Thylakoid membranes (50 mg protein) of WT and NdhL-His strains were solubilized with 0.25% n-dodecyl-β-D-maltoside and subjected to Ni<sup>2+</sup>-affinity chromatography according to [21]. The samples were taken before application to the Ni<sup>2+</sup> column, from the flow through fraction and from the eluted fraction.

### 2.3. Electrophoresis and protein identification

Protein complexes (7 μg protein) purified by Ni<sup>2+</sup>-affinity chromatography were loaded on 4.5–11% polyacrylamide gradient blue-native (BN) gel (Hoefer Mighty Small mini-vertical unit), and ran at 4 °C for 5 h. Solubilized thylakoid membranes (150 μg protein) were mixed with 1/40 volume of BN-gel sample buffer [22] and ran in the same kind of BN gel. The BN gel lanes were cut, solubilized and loaded onto a 1-mm-thick 14% SDS gel with 6 M urea [23]. The proteins in 2-D gels were visualized by silver staining.

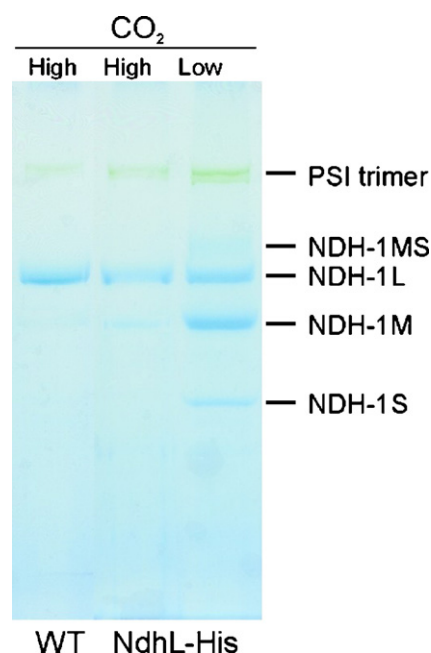


Fig. 1. Blue-native-PAGE of NDH-1 complexes of *Thermosynechococcus elongatus* WT and the NdhL-His strain grown under low and high CO<sub>2</sub> concentration. Isolated thylakoids were solubilized with 0.25% n-dodecyl-β-D-maltoside and the complexes purified by Ni<sup>2+</sup> affinity chromatography. For nomenclature and identification of the protein complexes, see also [21].

## 2.4. Electron microscopy

EM was performed as described in [24]. In short, EM specimens were prepared on glow-discharged carbon-coated grids, using 2% uranyl acetate as a negative stain. EM was performed on a Philips FEG20 electron microscope. Semi-automated data acquisition was used to record a total of 732, 2048 × 2048 pixel images at 66,850× magnification with a Gatan 4000 SP 4K slow-scan CCD camera. The step size (after the binning) was 30 μm, corresponding to a pixel size of 4.5 Å at the specimen level and projections were selected for single particle averaging [25] with Groningen Image Processing software. The average defocus of the micrographs was −300 μm. The images were corrected for CTF but processed without spatial filtering.

Projections were aligned by multi-reference alignment and aligned images were subjected to multivariate statistical analysis (MSA). After MSA, particles were classified and summed, and class sums were used in a next cycle of multi-reference alignment, MSA and classification. Final sums within homogeneous classes were obtained by reference-free alignment procedures [26]. Resolution was calculated by Fourier-ring correlation with the 3σ threshold as a criterion [24].

## 3. Results

### 3.1. Purification of NDH-1 complexes by Ni<sup>2+</sup> affinity chromatography

Membrane protein complexes of WT and NdhL-His strains grown at high and low CO<sub>2</sub> were purified by Ni<sup>2+</sup> affinity chromatography and applied to BN gel electrophoresis (Fig. 1).

The number and yield of protein complexes varied depending on the strains and growth conditions. Based on mass spectrometry (MS) analysis, the green band present in all lanes was recognized as PSI trimer and was present as contamination. As to NDH-1 complexes, WT cells grown at high CO<sub>2</sub> contained only one major band, the NDH-1L complex [21]. The NdhL-His strain grown at high CO<sub>2</sub> exhibited an additional band, which was identified as the NDH-1M complex. In the NdhL-His strain grown at low CO<sub>2</sub>, two more protein complexes were eluted from Ni<sup>2+</sup> column, and were identified as NDH-1S and NDH-1MS complexes (Fig. 1).

To elucidate the purified proteins, we took the crude thylakoid sample, the through fraction of Ni<sup>2+</sup> column as well as the purified complexes of the NdhL-His strain grown at low CO<sub>2</sub>, and applied them to BN/SDS-PAGE (Fig. 2). All major thylakoid protein complexes can be seen in the crude solubilized membranes, including PSI, PSII, cytochrome b<sub>6</sub>f, ATP synthase and the NDH-1 complexes (Fig. 2A). After passing the Ni<sup>2+</sup> column, there were almost no NDH-1 complexes present in the through fraction, whereas all other major protein complexes were present (Fig. 2B). This indicated that the binding of NDH-1 complexes to the Ni<sup>2+</sup> column was efficient and specific. Indeed, the elute fraction contained mainly the different forms of NDH-1 complexes (NDH-1L, NDH-1M, NDH-1S and NDH-1MS), and some PSI trimers as a contaminant (Fig. 2C). The subunits of NDH-1

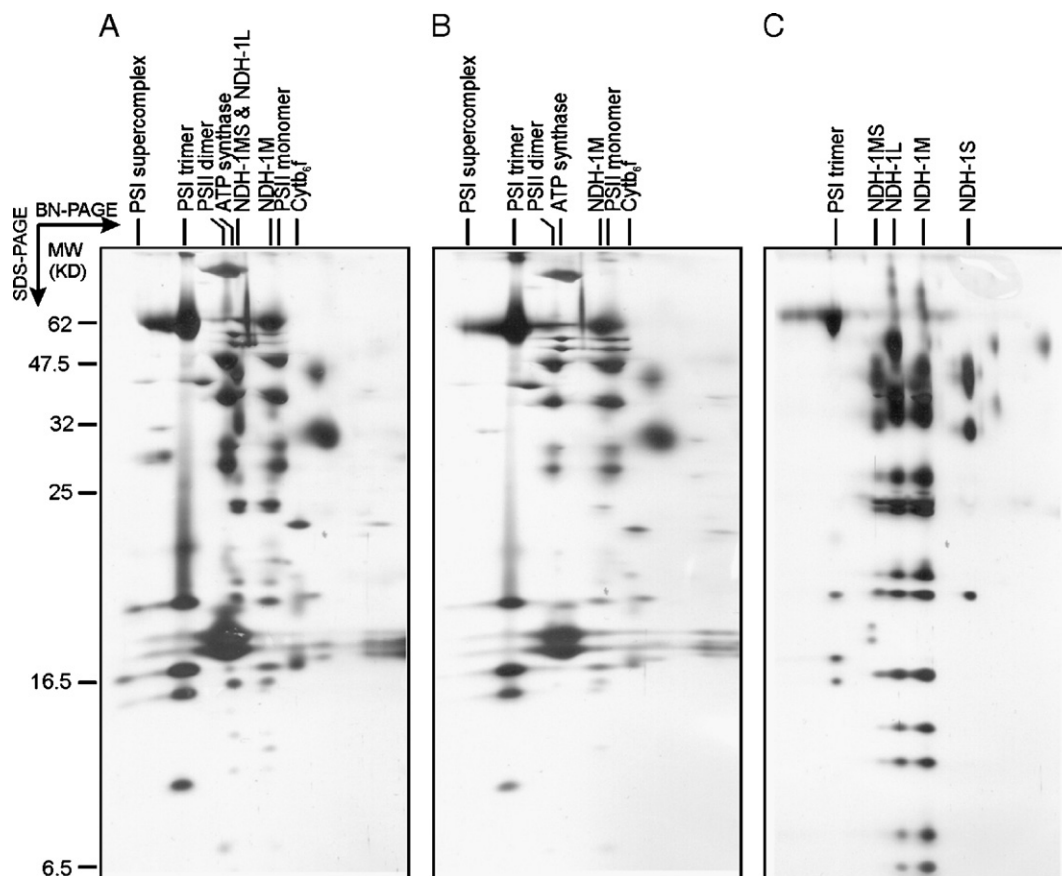


Fig. 2. Analysis of the NDH-1 complexes of the NdhL-His strain grown at low CO<sub>2</sub> conditions before and after purification by Ni<sup>2+</sup> affinity chromatography. The solubilized thylakoid membrane (A), the through fraction after Ni<sup>2+</sup> column (B), and the fraction eluted from the column (C) were collected, and subjected to BN/SDS-PAGE. Identification of the complexes and protein subunits is based on [21].



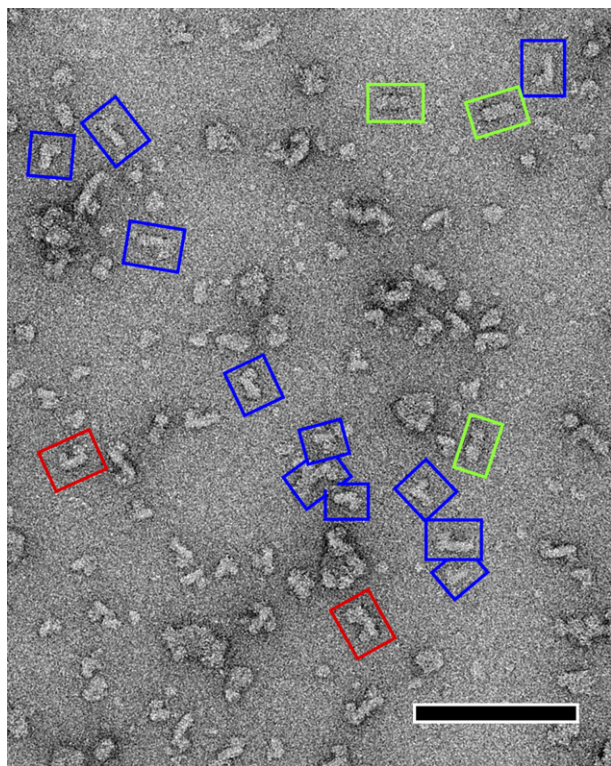


Fig. 3. TEM image of a negatively stained preparation of the NdhL-His strain grown at low CO<sub>2</sub> conditions after purification by Ni<sup>2+</sup> affinity chromatography. Side-view projections of L-shaped NDH-1 complexes (blue boxes), as well as top-view projections are indicated (green boxes). Two side views of U-shaped NDH-1 complexes are marked (red boxes). The space bar is 100 nm.

complexes (Fig. 2C) include NdhA-C, NdhD1,-E,-F1 and NdhG-O in NDH-1L; NdhA-O except NdhD1 and NdhF1 in NDH-1M; NdhD3, F3, CupA and Tli0220 in NDH-1S, and all subunits of NDH-1M and NDH-1S in NDH-1MS (for MS identification see [21]).

### 3.2. Electron microscopy

In order to investigate the structure of the multifunctional NAD (P)H dehydrogenase type 1 (NDH-1) complexes from cyanobacteria, we grew the wild type and a His-tagged NdhL mutant of *Thermosynechococcus elongatus* under different CO<sub>2</sub> conditions, and studied their purified membrane protein complexes. A large data set of about 25,000 side- and top-view projections was analysed by single particle electron microscopy (Fig. 3). In a subset of 15,000 projections of His-tag purified NDH-1 particles from *Thermosynechococcus* grown under low amount of CO<sub>2</sub> (“low CO<sub>2</sub>”), we can distinguish three different side-view projections (Fig. 4A–C). They all have a similar vertically oriented peripheral arm, but the membrane-embedded arm has a variable length. The largest particle is interpreted to be NDH-1L, because an almost identical type of projection was exclusively found in a set of 3,500 projections from WT NDH-1 (Fig. 3D). This WT NDH-1 could be purified to homogeneity, which is possibly more stable than the His-tagged NDH-1 construct. In a

set of 6000 projections of His-tag purified *Thermosynechococcus* NDH-1 particles grown under high amount of CO<sub>2</sub> the same type of NDH-1L projection is present (Fig. 4F). It is identical to the one of Fig. 4A at the current resolution of about 18 Å. The high CO<sub>2</sub> set also contained two smaller particles (Fig. 4G, H), similar to those of the batch of NDH-1 from the low CO<sub>2</sub>, though in much lower numbers. A low number of projections implies that the averaged images are still rather noisy and have lower resolution. Because of a lower resolution it is not possible to determine whether these particles are really identical or just similar. In the latter case they might also be (slightly) different in protein composition. The high CO<sub>2</sub> set also contained particles which we interpret as top view projections of NDH-1 (Fig. 4I), as well as smaller rod-like fragments. Some of them are 30–40% shorter than the top-view projection of Fig. 4I. Such top-view projections were not found in the low CO<sub>2</sub> batch, but this is not necessarily a matter of structural difference. The occurrence and frequency of various views, such as top- and side views, is strongly dependent on the properties of the carbon support film. For instance, in a previous set of NDH-1 particles, found as a contaminant of Photosystem II [24], we found several particles in another type of side-view position, with different handedness (Fig. 4J). This view was not found in the current sets.

In addition to NDH-1L and the smaller particles, we observed two types of NDH-1L particles with additional protein masses attached. One has a second hydrophilic arm at the tip of the membrane-embedded arm (Fig. 4E). This particle was present with low frequency (0.5–0.6%) in the high- and low CO<sub>2</sub> batch (Table 1), and is named NDH-1U because of its U-shaped form. The other one has a substantially longer peripheral arm. The overall abundance, however, was even lower (0.2%; Table 1). The additional mass appeared to be present in two positions (Fig. 4K, L). These particles have a long peripheral arm reminiscent of that of mitochondrial complex I, as in *Arabidopsis thaliana* (Fig. 4M) and many other species. It should be noted that in *Arabidopsis* some of the complex I particles may lose a part of the peripheral arm (Fig. 4N), and the resulting fragments appear to have a peripheral arm like those observed here for over 99% of the *Thermosynechococcus* NDH-1 particles.

### 4. Discussion

We have for the first time characterized the low-resolution structure of cyanobacterial NDH-1 complexes by electron microscopy and single particle averaging. The overall L-shape of the largest particle is in line with previous studies on the related complex I. It strongly resembles complex I from the fungus *Neurospora crassa* [17], the yeast *Yarrowia lipolytica* [27] and the eubacterium *Aquifex aeolicus* [28], except for the outer part of the peripheral arm which is lacking in the cyanobacterial projection maps (Fig. 4A, D, F). The similarity with the complex from bovine mitochondria is lower, because this complex has a more bulky peripheral arm [19]. The shorter peripheral arm of *Thermosynechococcus* is in line with the fact that the products of the nuo-E, -F, and -G genes from *E. coli*, which are supposed to be located within the peripheral arm and which have counterparts in the above mentioned organisms,

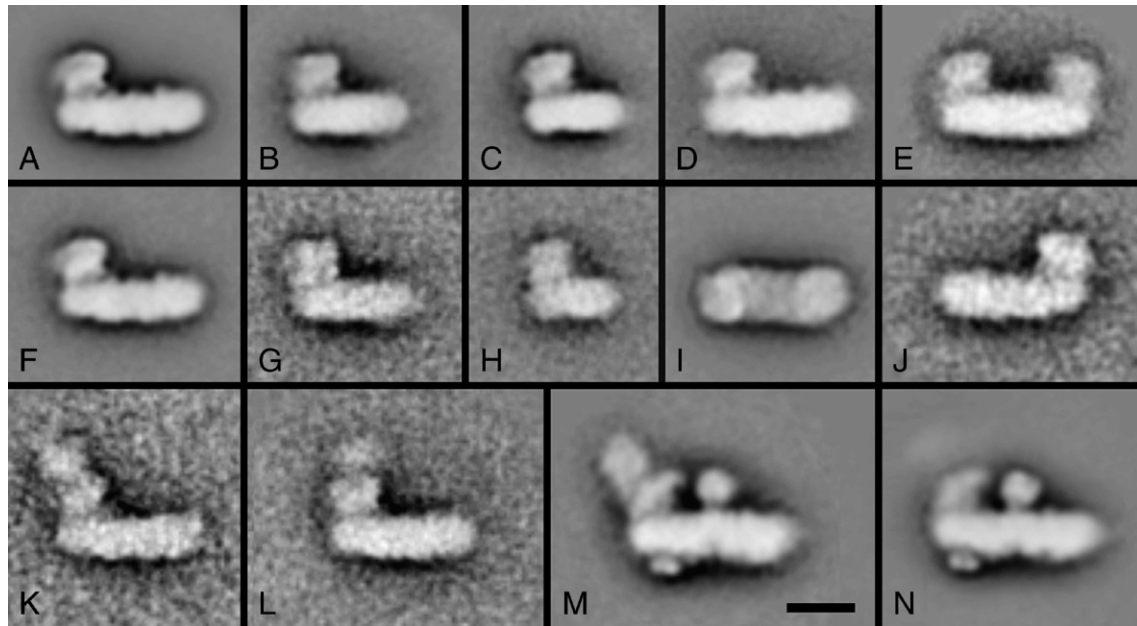


Fig. 4. Single particle electron microscopy of side- and top-view projections from different batches of NDH-1 complexes. (A–C) Three different types of projections found by classification of His-tag purified particles from the *Thermosynechococcus elongatus* NdhL-His strain grown under low amount of CO<sub>2</sub>. (D) Unique projection view of NDH-1, purified by chromatography from WT *Thermosynechococcus* grown under high amount of CO<sub>2</sub>. (E) U-shaped rare-view of NDH-1 complex from the *Thermosynechococcus elongatus* NdhL-His grown under low and high amount of CO<sub>2</sub>. (F–I) Five different types of side- and top-views of His-tag purified particles from the *Thermosynechococcus* NdhL-His strain, grown under high amount of CO<sub>2</sub>. (J) side view with different handedness, reproduced from [24]. (K, L) Rare views of NDH-1 complexes in all batches, in which an additional protein mass of 50–150 kDa lies at the top of the vertical arm. (M) Plant mitochondrial Complex I from *Arabidopsis* (reproduced from [33]). (N) Plant Complex I fragment from *Arabidopsis* lacking the NAD-oxidizing unit in the vertical arm (reproduced from [34]). The space bar is 10 nm.

have not been identified in cyanobacteria [2]. Homologs of the NuoE,-F, and -G subunits appear also to be absent in the plant mitochondrial Ndh (Complex I) complex [29]. Interestingly, in a small number of projections we found a larger peripheral arm, similar in size and shape to that found in the organisms mentioned above (Fig. 4K, L). These particles were present in both the low- and high CO<sub>2</sub> batches. This indicates that cyanobacteria like *Thermosynechococcus* must have protein(s) which are structural homologs of NuoE,-F, and -G. In our interpretation these proteins are only loosely associated, given the small fraction (0.2%) in the data set and the fact that dissociation of this part of the peripheral arm was also observed in plant mitochondrial Complex I from *Arabidopsis* (see Fig. 4N) and in Complex I from the alga *Polytomella* [30]. It should be noted that the *E. coli* NuoE,-F, and -G subunits comprise the NADH dehydrogenase fragment of complex I. It would of course be of interest to speculate on the nature of the additional mass in the NDH-1 complex. However, it is still uncertain what the electron donor for cyanobacterial NDH-1 is. It has been

suggested that instead of NADH, NADPH or ferredoxin might serve as the electron donor [30]. Our EM data indicate that the mass of the additional protein(s) on the top of the vertical arm, estimated from its surface area, is between 50 and 150 kDa. (This estimation based on comparison with electron microscopy maps of a couple of dozen well-defined multi-subunit proteins from which the high-resolution structure and hence also the mass is available). Ferredoxin has a mass of about 10 kDa and therefore is not a likely candidate to associate to NDH-1, as has been suggested [23].

The nature of NDH-1U, the U-shaped NADH-1 complex with a second hydrophilic arm at the tip of the membrane-embedded arm (Fig. 4E) is also intriguing. This particle was present with low frequency (0.5–0.6%) in the high- and low CO<sub>2</sub> batch (Table 1). Although it has not been observed before by any EM study of complex I we think that this U-shaped particle is not an artefact but that its frequency is only low because it is labile. Recently we performed image analysis of the complete set of large membrane proteins of *Thermosynechococcus* prepared for EM without any purification step after detergent solubilization with digitonin. Classification of projections indicated that at least 50% of the NDH-1 particles has a second hydrophilic arm, in a way as present in Fig. 4E (I.M. Folea and E.J. Boekema, unpublished results). Although a protocol to purify such particles has not yet been developed, it appears that with slight modifications of the currently applied purification scheme such particles are likely not to be obtained in sufficient yield to attempt assignment of its composition. Interestingly, the group of Nixon also found evidence for a

Table 1  
Relative numbers of NDH-1 complexes of the His-tag *Thermosynechococcus* NdhL-His strain grown under high- and low CO<sub>2</sub> levels

	number of single particles analysed	NDH-1L	NDH-1I	NDH-1M	NDH-1U	NDH-1X
High CO <sub>2</sub>	6.000	91.2%	4.7%	3.4%	0.5%	0.2%
Low CO <sub>2</sub>	15.000	51.7%	32.8%	14.7%	0.6%	0.2%

The numbers were found by statistical analysis of single particle projections.



smaller and a much larger (or extra) peripheral arm from a proteomic analysis of NDH-1 fractions [31]. They found two particles by blue-native PAGE, differing by 130 kDa. From gel staining patterns, this difference was interpreted as being caused by the presence of a second set of the hydrophilic subunits H, I, J, K and slr1623.

For the assignment of the electron microscopy projections which are smaller than NDH-1L, we should take into account their relative masses on blue-native PAGE and their protein composition determined after two-dimensional gel analysis and mass spectrometry [22,23]. The four different NDH-1 complexes found previously at low CO<sub>2</sub> have masses of about 490 kDa (NDH-1 L), 350 kDa (NDH-1M), 200 kDa (NDH-1S<sub>1</sub>) and 140 kDa (NDH-1S<sub>2</sub>). The two fragments found by electron microscopy analysis differ by about 15% and 30% in surface area from the largest NDH-1L particle, respectively. Since the complex previously assigned as NDH-1M is about 30% smaller in mass than NDH-1L, it is probably similar to the one presented in Fig. 4C, H. If this interpretation is correct the particle of Fig. 4B, G would represent one of intermediate size. The difference between the NDH-1L and NDH-1M complexes is the presence of the NdhD1 and NdhF1 subunits. If only one of them (either NdhD1 or NdhF1) were absent, it could very well explain the presence of the intermediate complex (assigned as NDH-1I), which differs only by 15%. The fact that NdhD1 and the C-terminal fragment of NdhF1 can be easily removed from the NDH-1 complexes has been observed by others [31]. They found a hydrophobic NDH-1 fragment, lacking all the peripheral arm subunits from which NdhD1 and NdhF1 were also missing.

The fact that the NDH-1M and NDH-1I complexes are relatively abundant under low CO<sub>2</sub> is also obvious from the statistical analysis. Numbers of projections from the single particle analysis are compared in Table 1, which shows that there is a 6-fold difference in abundance of the NDH-1M and NDH-1I views between high- and low CO<sub>2</sub> growth conditions. This is in good agreement with the results presented in [23]: under low CO<sub>2</sub> the NDH-1M complex, which is a prerequisite for CO<sub>2</sub> uptake, increases substantially whereas the NDH-1L contents remain practically unchanged. It should also be noticed that we did not focus on the characterization of the smallest particles present in the set, because they are more difficult to analyse. Some of them are rod-like projections, 30–40% shorter than the top view projection of NDH-1 (Fig. 4I) and present in about 5% abundance. It is not possible to assign to them to any specific complex, but some could represent NDH-1S, which is induced at low CO<sub>2</sub> condition [23]. Others could represent the NDH-1 fragment, lacking all the peripheral arm subunits and NdhD1 and NdhF1, mentioned above [31].

Single particle EM is a very suitable method to analyse the heterogeneity of proteins such as cyanobacterial NDH-1, because it can handle heterogeneous data sets of projections. The general conclusion of our EM analysis is that cyanobacterial NDH-1 appears to be both unexpectedly complex and quite labile concerning the two hydrophilic arms. This resembles the overall low stability of the Ndh complex, which is the homolog of NDH-1 in plant chloroplasts [32]. This complex likely has a short hydrophilic arm as well because it appears to lack homologs of the

NuoE, -F, and -G subunits [29]. Due to its instability and low yield it was not yet studied by electron microscopy. The isolated complex I particles from *Arabidopsis* and *Polytomella* mitochondria do have the homologs of the NuoE, -F, and -G subunits and show a much higher percentage of intact hydrophilic arms than the low numbers (0.2%) observed here for the cyanobacterial complex. Even in the above mentioned pilot study (I.M. Folea and E.J. Boekema, unpublished) of non-purified NDH-1 this percentage is not significantly higher, indicating that the NDH-1 particle with a short hydrophilic arm probable does not have this extension attached in stoichiometric ratios. On the other hand, the U-shaped particle NDH-1U with a second hydrophilic arm could be the functional complex in cyanobacteria. Assignment of its composition by mass spectroscopy and possible further characterization, however, depends on its capacity to become purified. Although  $\beta$ -dodecyl maltoside is the most commonly applied detergent for purifying membrane proteins from the photosynthetic membrane of cyanobacteria, it clearly fails in keeping the second hydrophilic arm intact. Thus new purification schemes need to be developed, replacing  $\beta$ -dodecyl maltoside by another detergent like digitonin. If such schemes cannot retain the second hydrophilic arm the determination of subunit composition could only be achieved by gold-labelling of introduced His-tags in combination with single particle analysis [33] on non-purified material.

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## References

- [1] H. Kotani, S. Tabata, Lessons from sequencing of the genome of a unicellular cyanobacterium, *Synechocystis* Sp. PC6803, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49 (1998) 151–171.
- [2] T. Friedrich, K. Steinmüller, H. Weiss, The proton pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts, *FEBS Lett.* 367 (1995) 107–111.
- [3] M. Shibata, H. Ohkawa, T. Kaneko, H. Fukuzawa, S. Tabata, A. Kaplan, T. Ogawa, Distinct constitutive and low-CO<sub>2</sub>-induced CO<sub>2</sub> uptake systems in cyanobacteria: genes involved and their phylogenetic relationship with homologous genes in other organisms, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 11789–11794.
- [4] M. Shibata, H. Katoh, M. Sonoda, H. Ohkawa, M. Shimoyama, H. Fukuzawa, A. Kaplan, T. Ogawa, Genes essential to sodium-dependent bicarbonate transport in Cyanobacteria: function and phylogenetic analysis, *J. Biol. Chem.* 277 (2002) 18658–18664.
- [5] P. Tamagnini, R. Axelsson, P. Lindberg, F. Oxelfelt, R. Wünschiers, P. Lindblad, Hydrogenases and hydrogen metabolism of cyanobacteria, *Microbiol. Mol. Biol. Rev.* 66 (2002) 1–20.
- [6] G.D. Price, D. Sültemeyer, B. Klughammer, M. Ludwig, M.R. Badger, The functioning of the CO<sub>2</sub> converting mechanism in several cyanobacterial strains: a review of general physiological characteristics, genes, proteins and recent advances, *Can. J. Bot.* 76 (1998) 973–1002.
- [7] H. Mi, T. Endo, U. Schreiber, T. Ogawa, K. Asada, Electron donation from cyclic and respiratory flows to the photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803, *Plant Cell Physiol.* 33 (1992) 1233–1237.
- [8] H. Mi, T. Endo, T. Ogawa, K. Asada, Thylakoid membrane-bound, NADPH-specific pyridine-nucleotide dehydrogenase complex mediates cyclic

- electron-transport in the cyanobacterium *Synechocystis* SP PCC-68038, *Plant Cell Physiol.* 36 (1995) 661–668.
- [9] H. Ohkawa, H.B. Pakrasi, T. Ogawa, Two types of functionally distinct NAD(P)H dehydrogenases in *Synechocystis* sp. Strain PCC6803, *J. Biol. Chem.* 275 (2000) 31630–31634.
- [10] H. Ohkawa, M. Sonade, H. Katoh, T. Ogawa, The use of mutants in the analysis of the CO<sub>2</sub>-concentrating mechanism in cyanobacteria, *Can. J. Bot.* 76 (1998) 1035–1042.
- [11] B. Klughammer, D. Sultemeyer, M.R. Badger, G.D. Price, The involvement of NAD(P)H dehydrogenase subunits, NdhD3 and NdhF3, in high-affinity CO<sub>2</sub> uptake in *Synechococcus* sp. PCC7002 gives evidence for multiple NDH-1 complexes with specific roles in cyanobacteria, *Mol. Microbiol.* 32 (1999) 1305–1315.
- [12] S. Maeda, M.R. Badger, G.D. Price, Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO<sub>2</sub> hydration in the cyanobacterium *Synechococcus* sp PCC7942, *Mol. Microbiol.* 43 (2002) 425–435.
- [13] M. Volokita, D. Zenvirth, A. Kaplan, L. Reinhold, Nature of the inorganic carbon species actively taken up by the cyanobacterium *Anabena variabilis*, *Plant. Physiol.* 76 (1984) 599–602.
- [14] A. Kaplan, L. Reinhold, CO<sub>2</sub> concentrating mechanisms in photosynthetic microorganisms, *Annu. Rev. Plant Physiol. Mol. Biol.* 50 (1999) 539–570.
- [15] T. Ogawa, A. Kaplan, Inorganic carbon acquisition systems in cyanobacteria, *Photosynth. Res.* 77 (2003) 105–115.
- [16] G. Hofhaus, H. Weiss, K. Leonard, Electron microscopic analysis of the peripheral and membrane parts of mitochondrial NADH dehydrogenase (Complex I), *J. Mol. Biol.* 221 (1991) 1027–1043.
- [17] V. Guenebaut, R. Vincentelli, D. Mills, H. Weiss, K.R. Leonard, Three-dimensional structure of NADH-dehydrogenase from *Neurospora crassa* by electron microscopy and conical tilt reconstitution, *J. Mol. Biol.* 265 (1997) 409–418.
- [18] N. Grigorieff, Three-dimensional structure of bovine NADH: ubiquinone oxidoreductase (Complex I) at 22 Å in ice, *J. Mol. Biol.* 277 (1998) 1033–1046.
- [19] B. Böttcher, D. Scheide, M. Hesterberg, L. Nagel-Steger, T. Friedrich, A novel, enzymatically active conformation of the *Escherichia coli* NADH: ubiquinone oxidoreductase (Complex I), *J. Biol. Chem.* 277 (2002) 17970–17977.
- [20] A. Abdrakhmanova, V. Zickermann, M. Bostina, M. Radermacher, H. Schägger, S. Kerscher, U. Brandt, Subunit composition of mitochondrial complex I from the yeast *Yarrowia lipolytica*, *J. Mol. Biol.* 1658 (2004) 148–156.
- [21] P. Zhang, N. Battchikova, V. Paakkarinen, H. Katoh, M. Iwai, M. Ikeuchi, H.B. Pakrasi, T. Ogawa, E.-M. Aro, Isolation, subunit composition and interaction of the NDH-1 complexes from *Thermosynechococcus elongatus* BP-1, *Biochem. J.* 390 (2005) 513–520.
- [22] M. Herranen, N. Battchikova, P. Zhang, A. Graf, S. Sirpio, V. Paakkarinen, E.-M. Aro, Towards functional proteomics of membrane protein complexes in *Synechocystis* sp. PCC 6803, *Plant Physiol.* 134 (2004) 470–481.
- [23] P. Zhang, N. Battchikova, T. Jansen, J. Appel, T. Ogawa, E.-M. Aro, Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/ NdhF3/CupA/Sll1735 in *Synechocystis* sp PCC 6803, *Plant Cell* 16 (2004) 3326–3340.
- [24] A.A. Arteni, M. Nowaczyk, J. Lax, R. Kouřil, M. Rögner, E.J. Boekema, Single particle electron microscopy in combination with mass spectrometry to investigate novel complexes of membrane proteins, *J. Struct. Biol.* 149 (2005) 325–331.
- [25] G. Harauz, E.J. Boekema, M. van Heel, Statistical analysis of electron micrographs of ribosomal subunits, *Methods Enzymol.* 164 (1988) 35–49.
- [26] P. Penczek, M. Radermacher, J. Frank, Three-dimensional reconstruction of single particles embedded in ice, *Ultramicroscopy* 40 (1992) 33–53.
- [27] R. Djafarzadeh, S. Kerscher, K. Zwicker, M. Radermacher, M. Lindahl, H. Schägger, U. Brandt, Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*, *Biochim. Biophys. Acta* 1459 (2000) 230–238.
- [28] G. Peng, G. Fritzch, V. Zickermann, H. Schägger, R. Mentle, F. Lottspeich, M. Bostina, M. Radermacher, R. Huber, K.O. Stetter, H. Michel, Isolation, characterization and electron microscopic single particle analysis of the NADH: ubiquinone (ComplexI) from the hyperthermophilic eubacterium *Aquifex aeolicus*, *Biochemistry* 42 (2003) 3032–3039.
- [29] P. Rumeau, N. Bécuwe-Linka, A. Bewly, M. Louwagie, J. Garin, G. Peltier, New subunits NDH-M, -N, and -O, encoded by nuclear genes, are essential for plastid Ndh complex functioning in higher plants, *Plant Cell* 17 (2005) 219–232.
- [30] S. Sunderhaus, N. Dudkina, L. Jansch, J. Klodmann, J. Heinemeyer, M. Perales, E. Zabaleta, E. Boekema, H.P. Braun, Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants, *J. Biol. Chem.* 281 (2006) 6482–6488.
- [31] P. Prommeeenate, A.M. Lennon, C. Markert, M. Hippler, P.J. Nixon, Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803: identification of two new ndh gene products with nuclear-encoded homologues in the chloroplast Ndh complex, *J. Biol. Chem.* 279 (2004) 28165–28173.
- [32] L.A. Sazanov, P.A. Burrows, P.J. Nixon, The plastid *ndh* genes code for an NADH-specific dehydrogenase: isolation of a complex I analogue from pea thylakoid membranes, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 1319–1324.
- [33] C. Büchel, E. Morris, E. Orlova, J. Barber, Localisation of the PsbH subunit in photosystem II: a new approach using labelling of His-tags with a Ni(2+)-NTA gold cluster and single particle analysis, *J. Mol. Biol.* 312 (2001) 371–379.
- [34] N.V. Dudkina, H. Eubel, W. Keegstra, E.J. Boekema, H.P. Braun, Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III, *Proc. Natl. Acad. Sci.* 102 (2005) 3225–3229.